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Our research is structured to investigate of the role of polychlorinated biphenyls (PCBs) in breast cancer induction. Progress is reported in several areas: 1) Microsomes prepared from human breast tissue catalyze the hydroxylation of lower chlorinated PCBs to mono- and di-hydroxy metabolites. 2) Several mono- and di-hydroxy metabolites of PCBs are estrogenic in a human breast cell assay, the MCF-7 Focus assay, with the para hydroxylated the most active. 3) To detect alterations in nuclear proteins, liver nuclear extracts were analyzed by electrophoretic mobility shift assays. 4-Chloro-, 2,4,4'-trichloro, and 2,2',4,4',5,5'-hexachlorobiphenyl treatment of Sprague-Dawley rats resulted in a large increase in protein binding to a consensus AP-1 element. The latter two PCBs also showed STAT binding activity to a consensus GAS element. 4) During the oxidation of di-hydroxy PCB metabolites, superoxide produced is detected by following the reduction of Nitro Blue Tetrazolium and measuring DNA strand breaks. 5) To determine if PCB quinones are able to participate in redox events, a test system employing acetylated cytochrome c was employed. The rate of cytochrome c reduction, in the presence and absence of superoxide dismutase, indicated that all PCB quinones, except one, produce superoxide. 6) An assay for the determination of oxidized DNA bases, especially 8-oxodeoxyguanosine has been established.

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Date

TABLE OF CONTENTS

INTRODUCTION

Our research is aimed at the investigation of the effects of polychlorinated biphenyls (PCBs) in breast cancer. PCBs are industrial chemicals which persist in our environment. The lipophilicity of PCBs and their tendency to bioaccumulate in adipose tissue and breast milk raise concern about the health risks associated with exposure to PCBs and related compounds. Commercial PCB mixtures are complete carcinogens, producing hepatocellular carcinomas in rats and mice, but the mechanisms by which they do so have not been determined. We and others have shown that higher halogenated PCBs (especially, tetra-, penta-, and hexa-chlorinated biphenyls) act as promoters of carcinogenesis, but their initiating or DNA damaging activity has not been conclusively demonstrated. In our original proposal we presented considerable data to support the concept that the lower halogenated biphenyls may be activated by hepatic and breast (milk) enzymes to oxygenated species that are electrophilic and bind to DNA. Of particular interest were the quinone metabolites. Our data showed that PCBs are metabolized to dihydroxy metabolites, that these can be oxidized by peroxidases (including lactoperoxidase) and prostaglandin synthase to quinones, that these PCB-quinones are strong electrophiles that react with both sulfur and nitrogen nucleophiles, including nucleotides and DNA. We offered preliminary observations that supported the concept that PCB quinones redox cycle.

To build on these observations, we proposed (i) to determine if PCBs that accumulate in breast tissue are converted to dihydroxy metabolites that can be oxidized by breast tissue subcellular fractions and lactoperoxidase to PCB quinones, (ii) to characterize the DNA-adducts of breast-specific PCBs with regard to the specific metabolites formed and nucleotides involved and to identify the chemical structure of the adducts, (iii) to determine the biological consequences of DNA-adduct formation by PCB metabolites, including detection of single- and double-strand breaks, analysis of sites that block in vitro DNA synthesis and analysis of mutations, and (iv) to employ in vivo models to identify PCB adducts and mutations in the breast, and to investigate the possibility of using DNA adduction for human biomonitoring purposes by detecting DNA-reacting metabolites in serum and breast milk. These studies address our working hypothesis that PCB congeners that accumulate in the breast may be metabolized in this tissue to electrophiles, especially quinones, which then react with critical cellular targets, including DNA, and that these reactions lead to mutagenic events resulting in neoplastic change. Our project therefore addresses the question of the possible mechanisms of PCB carcinogenicity, with emphasis on the human breast as target organ.

BODY OF THE REPORT

1. HUMAN TISSUES AND METABOLISM

In previous years we have invested considerable time and effort in exploring the metabolic activity of rat liver microsomes toward individual PCB congeners. Our studies of the metabolism of 4-chlorobiphenyl in rat liver microsomes isolated from rats treated with selected inducers have been published (McLean et al., 1996a). The absolute identification of metabolites was only possible after our synthesis of authentic standards (Bauer et al., 1995). Please note a representative chromatogram (Figure 1) of the extracted and silylated metabolites of 4-chlorobiphenyl below.

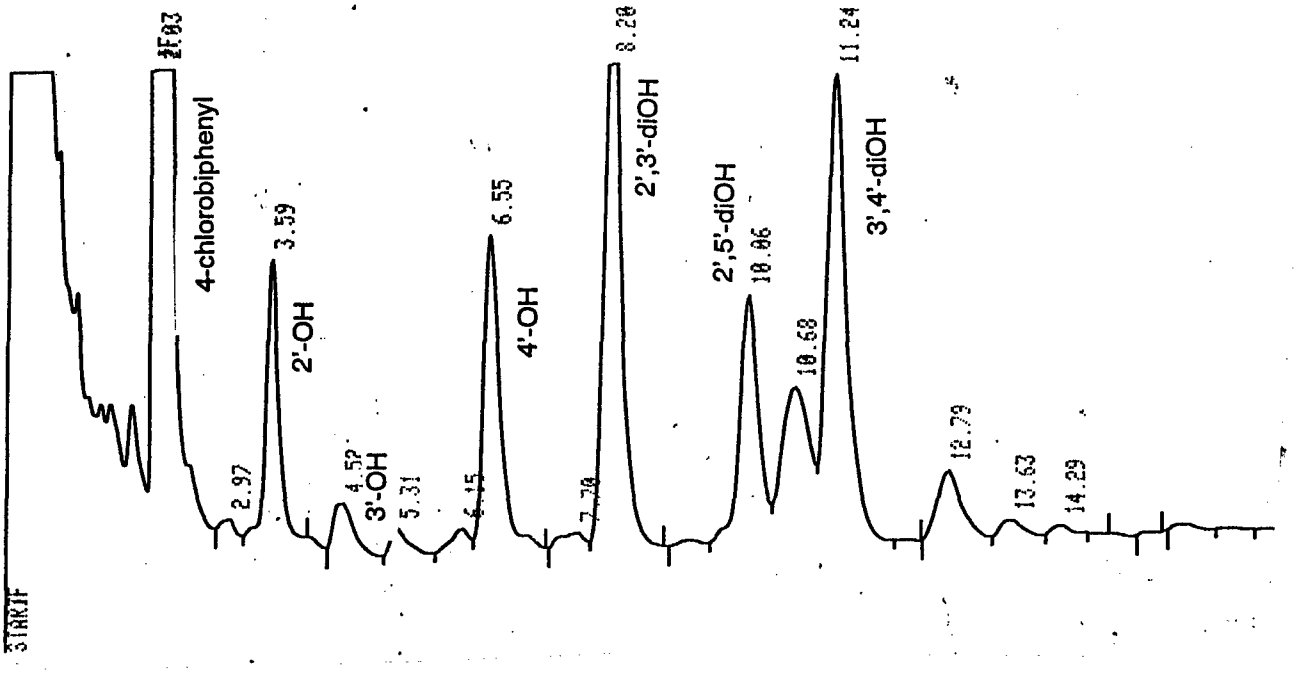


Figure 1: Gas chromatogram with flame ionization detection of extracted and silylated metabolites of 4-chlorobiphenyl . The parent PCB was incubated with rat liver microsomes, isolated from rats treated with phenobarbital and 3-methylcholanthrene, and a NADPH-regenerating system. The PCB 4-chlorobiphenyl and three mono-hydroxy metabolites (2'-OH, 3'-OH and 4'-OH) as well as three di-hydroxy metabolites (2',3'-diOH, 2',5'-diOH, and 3',4'-diOH) were detected by comparison with authentic standards. A Hewlett-Packard 5890A gas chromatograph fitted with an HP1 methyl silicone gum column (5m x 0.53mm x 2.65μm film thickness) was used to separate the metabolites. The injector and flame ionization detector temperatures were 225°C and 300°C, respectively. The carrier gas helium was maintained at 10 psi head pressure. The temperature program was: initial temperature 140°C, held for one minute, ramped to 245°C at 5°C per minute, and then held at 245°C for 6 minutes. Peaks were recorded with a HP-3392A integrator.

Our current work addresses the question of whether human breast microsomes are capable of catalyzing the hydroxylation of PCBs and identifying the metabolic products. In the past several months we have obtained several human breast tissue samples from two sources (in Alabama and in Ohio) and have prepared microsomes from them. Our initial attempts to quantify the catalytic activity of these human subcellular fractions toward a model PCB substrate are depicted below in Figure 2.

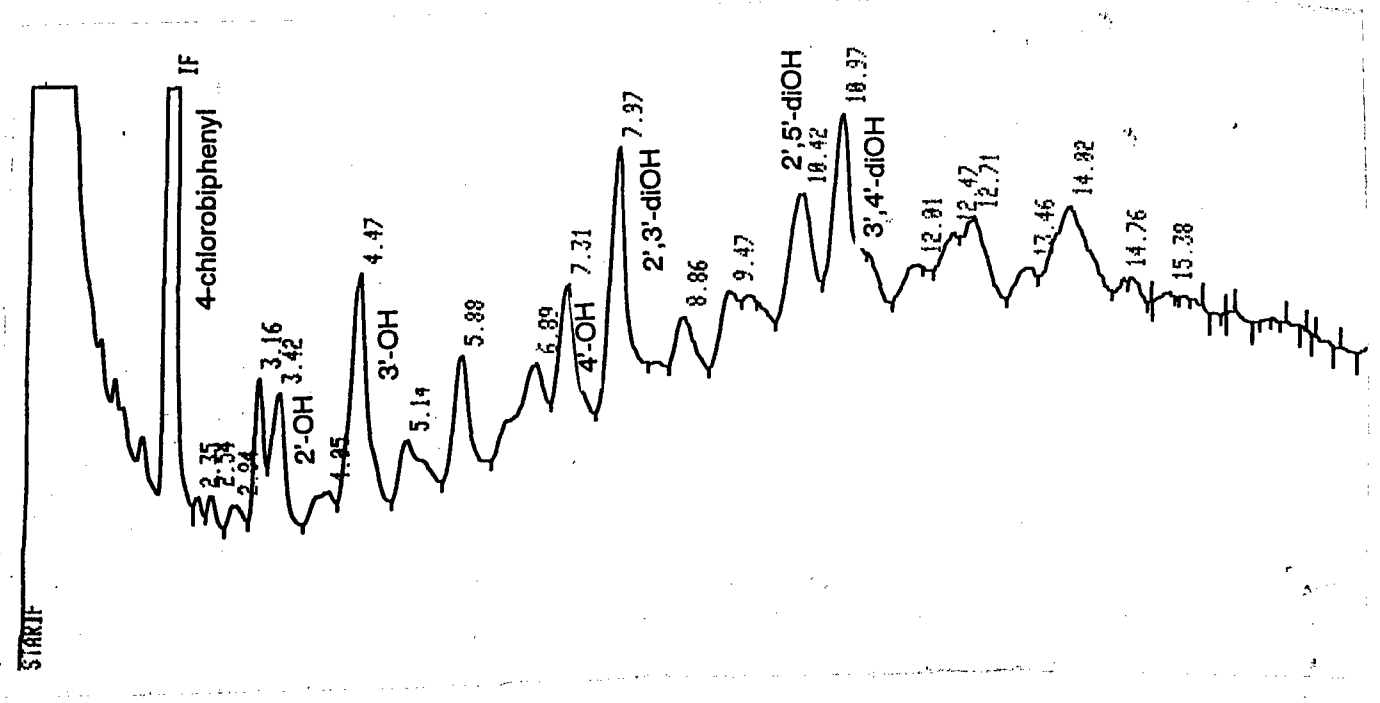


Figure 2: Gas chromatogram with flame ionization detection of extracted and silylated metabolites of 4-chlorobiphenyl . The parent PCB was incubated with microsomes, prepared from human breast tissue, and a NADPH-regenerating system. Our tentative assignment of structures and the gas chromatographic conditions are as stated in the legend to Figure 1.

Considerable time and effort were expended in developing the optimal extraction conditions, optimized to extract the PCB metabolites, while leaving behind fatty acids and lipids. Improvement of our methods in this area as well as further studies on the catalytic activity of human breast tissue are continuing.

2. HYDROXY METABOLITES AND ESTROGENICITY

Our successes in the synthesis of several mono- and di-hydroxy metabolites of PCBs have enabled us to explore the estrogenicity (and anti-estrogenicity) of these compounds through a collaboration with Dr. John Gierthy of the Wadsworth Center, New York State Department of Health in Albany. We were particularly interested in exploring the importance of the position of the hydroxyl group, since most studies had used PCBs hydroxylated in the para position only. We examined the effect of the position of the hydroxyl group on the activity of 2,5-dichlorobiphenyl (2,5-DCBP) and

3,4-DCBP. Estrogenicity and antiestrogenicity were tested in a human breast cell assay, the MCF-7 Focus assay, in which cells respond to 17-ß estradiol with post-confluent proliferation and formation of nodules or foci with an EC_{50} of 0.1 nM. Results show that at 5 μM 1) the PCB congener 2,5-DCBP was weakly estrogenic, whereas 3,4-DCBP was not, 2) neither of the parent congeners was antiestrogenic, 3) the para hydroxylated metabolites exhibited the greatest estrogenicity for each DCBP, 4) the meta hydroxyl metabolite of 2,5-DCBP was estrogenic, while the meta hydroxyl metabolite of 3,4-DCBP was not, 5) none of the hydroxylated 2,5-DCBPs was antiestrogenic, while 6) the 3,4-dichloro-3'-biphenylol was antiestrogenic. In conclusion, these results and others indicate that the estrogenicity of hydroxylated PCB metabolites is mediated through an estrogen receptor and modulated by the position of the hydroxyl group. The most active PCB metabolites are para hydroxylated and these exhibit less than one thousand fold the potency of the endogenous human estrogen, 17ß-estradiol. Progress on this collaborative research project has been reported at two conferences and in a proceedings chapter (Arcaro et al., 1997).

3. ACTIVATION OF AP-1 AND STAT TRANSCRIPTION FACTORS BY NON-COPLANAR PCBS

The promoting effects of PCBs have been extensively studied in a variety of two stage carcinogenesis models. However, the molecular mechanisms responsible for the promoting effects of PCBs have not been elucidated. The purpose of this study was to determine the effect of PCBs on DNA binding proteins involved in cell-proliferation and transformation. Male Sprague-Dawley rats were injected i.p. with either mono-, di-, tri-, tetra-, or hexachlorobiphenyls (300 μmols/kg/day) each day for 4 days and were sacrificed 4 hours after the last injection. To detect alterations in nuclear proteins that may potentially explain the tumor promoter activity of PCBs, liver nuclear extracts were analyzed by electrophoretic mobility shift assays (EMSAs). 4-Chloro-, 2,4,4'-trichloro, and 2,2',4,4',5,5'-hexachlorobiphenyl treatment of Sprague-Dawley rats resulted in a large increase in protein binding to a consensus AP-1 element. However, 3,4-dichloro- and 3,3',4,4'-tetrachlorobiphenyl treatment did not increase AP-transcription activity. Further analysis of the proteins binding to the AP-1 consensus sequence using antibodies specific for c-FOS, JUN-D and JUN-B indicate that the protein composition consists of JUN-B proteins. EMSA analysis of STAT binding activity to a consensus GAS element was compared in the various PCB treated liver nuclear extracts. STAT binding activity was 8 to 10-fold higher in nuclear extracts from 2,4,4'-trichloro- and 2,2',4,4',5,5'-hexachlorobiphenyl-treated animals. Analysis of the protein complex binding to the GAS element, using antibodies specific for STAT3, STAT5, and STAT6, indicate that the protein composition is made up of both STAT5 and STAT6 proteins. HepG2 cells transiently transfected with a luciferase reporter gene construct containing multiple STAT5 binding sites were treated with 2,4,4'-trichloro, and 2,2',4,4',5,5'-hexachlorobiphenyl. 2,4,4'-Trichlorobiphenyl stimulated a 4-5 fold increase in luciferase activity. However, enhanced luciferase activity did not occur with 2,2',4,4',5,5'-hexachlorobiphenyl treatment. These data demonstrate early molecular events that

may be responsible for the tumor promoting effects of PCBs. (These data are to be presented at the next Society of Toxicology annual meeting.)

4. SUPEROXIDE PRODUCTION: NBT ASSAY

The spontaneous or enzymatic oxidation of hydroquinone and catechol metabolites of PCBs is expected to result in the production of superoxide anion radicals (see Figure 3 below). Nitro blue tetrazolium (NBT) is reduced by superoxide to the insoluble blue formazan. One method to measure superoxide production is by measuring the formation of formazan from NBT spectrophotometrically at 540 nm. If not otherwise indicated, 90 μM NBT was incubated in a 10 mM phosphate buffer with 100 μM EDTA and 5 mM CTAB in a quartz cuvette with a final volume of 1 ml. The reaction was started by adding the PCB-metabolite to a final concentration of 100 μM. The increase of absorption at 540 nm was followed for 10 minutes.

To test superoxide production with dihydroxybiphenyl-metabolites, 0.01 U/ml lactoperoxidase and 0.5 mM H_2O_2 were added for enzymatic oxidation of the compound. 3,4-dichloro-3',4'-dihydroxybiphenyl did not produce formazan production under these conditions. With 3,4-dichloro- and 4-chloro-2',5'-dihydroxybiphenyl, a slow but steady increase in absorption was observed, indicating the production of about 6-9 nmoles superoxide in 10 minutes. The disappearance of the dihydroxy-metabolite and appearance of the quinone-metabolite was shown by comparing spectral profiles before and after incubation with those of known standards. When glutathione (GSH) was added to the incubations with the para-dihydroxy-metabolites in equimolar concentration, a fast and significant increase in formazan was observed within the first 4 minutes. The total superoxide production after 10 minutes was 1-3 fold higher when GSH was present.

Superoxide production by the para-quinone metabolites was tested without peroxidase/H_2O_2. 4-chloro- and 3,4-dichloro-2',5'biphenylquinone did not produce any superoxide under these conditions. When equimolar concentrations of GSH were added, however, about 30 and 42 nmoles superoxide, respectively, were produced within 10 minutes. These results show that para-dihydroxy-metabolites of PCBs can produce potentially harmful superoxide radicals during enzymatic oxidation. Quinone metabolites can be reduced by conjugation with GSH. The resulting conjugate can autoxidize and thereby form even more superoxide. As we will show below, this may have damaging effects on DNA.

5. SUPEROXIDE PRODUCTION: REDOX CYCLING

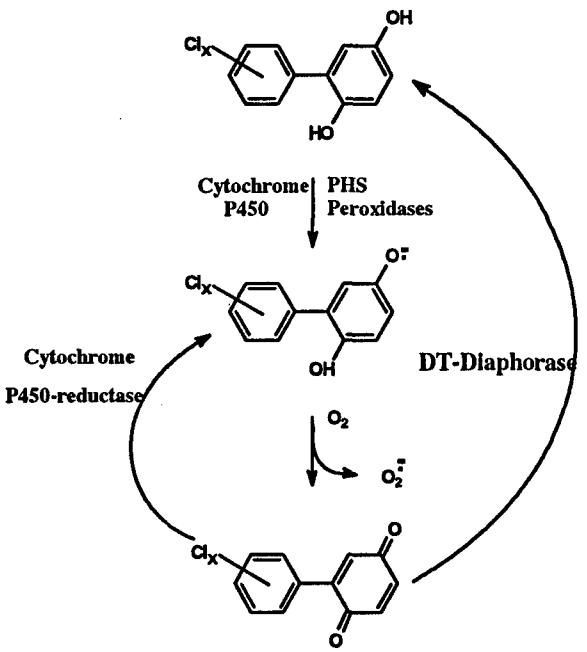
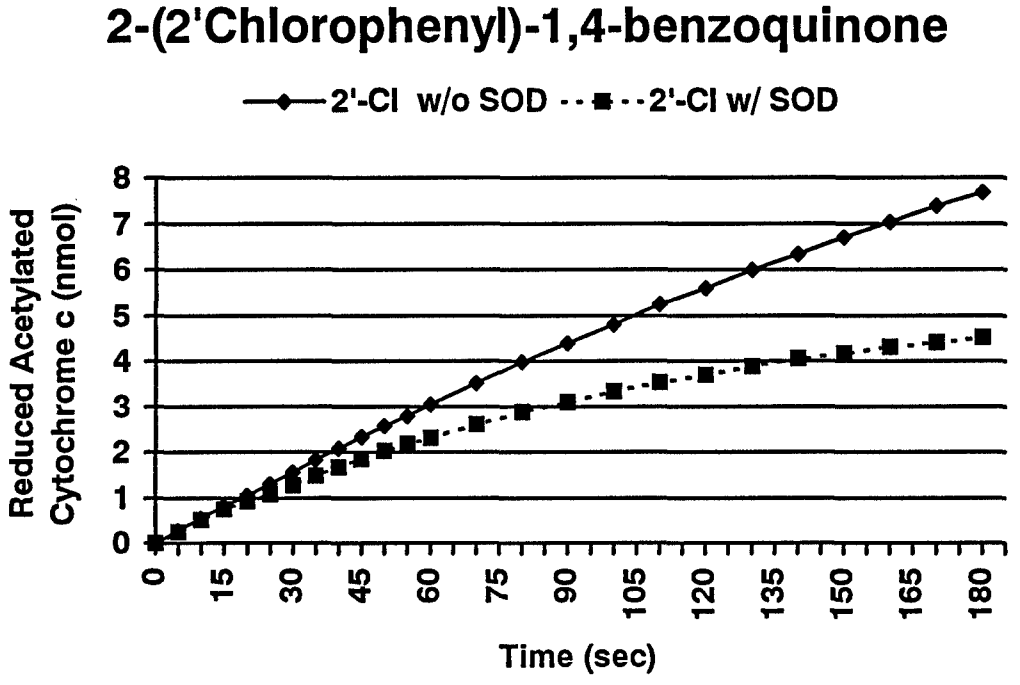


Figure 3

PCBs are considered problem compounds by the scientific community as well as the general public due to their stability and persistence in the environment, as well as their tendency to bioaccumulate in target tissues such as the liver or breast. Recent work in our laboratory demonstrates that PCBs may be metabolized to form dihydroxy metabolites, and that these metabolites may then autoxidize or may be enzymatically oxidized to semiquinones and/or quinones (McLean et. al., 1996a; Oakley et al., 1996a). These metabolites may then react with oxygen to form reactive oxygen species, such as superoxide free radicals (Figure 3).

To determine if PCB quinones are able to participate in redox events, we incubated liver microsomes, PCB quinones, a NADPH regenerating system, and cytochrome c as an *in vitro* system. We then measured the rate of cytochrome c reduction as an indicator of superoxide production. To confirm that superoxide free radicals were in fact being produced, we added superoxide dismutase (SOD) to the system. If superoxide were being produced, then superoxide would be scavenged by the SOD, and the rate of cytochrome c reduction would decrease, indicating that superoxide was a product in the PCB quinone-catalyzed redox cycling. The PCB quinones included in this experiment included mono-, di-, and tri-chlorinated compounds. Also included as baseline compounds were the 1,4-benzoquinone as well as the 2-phenyl-benzoquinone. A representative example of these data is shown in (Figure 4).



The data are summarized for all the quinone compounds tested in this system in Table 1. This table shows the difference in the rates of cytochrome c reduction in the presence and the absence of SOD. The data confirm that SOD does in fact decrease the rate of reduction, and indirectly shows that superoxide free radicals are a component in this reaction. There was one interesting outlier in this experiment, namely the 4'-chloro-2,5 quinone. SOD had no effect on the rate of cytochrome c reduction in the presence of this compound. We have no explanation at this time for this observation.

Table 1:

Compound:	"Total" Reduction of Cytochrome c	- "SOD"	= Difference (@ 180 sec)
1,4-Benzoquinone	6.294	3.778	2.516
2-Phenyl-Benzoquinone	8.352	4.705	3.647
2'-chloro-2,5-quinone	7.690	4.525	3.165
3'-chloro-2,5-quinone	6.402	4.248	2.154
4'-chloro-2,5-quinone	2.395	2.371	0.024
3',4'-chloro-2,5-quinone	4.392	2.936	1.456
3',5'-chloro-2,5-quinone	3.574	2.479	1.095
3',4',5'-chloro-2,5-quinone	5.439	4.019	1.420

These data were presented in part at the last Society of Toxicology Annual Meeting and are currently being written up for publication.

6. INDUCTION OF STRAND BREAKS BY PCB METABOLITES

Compounds that produce oxygen radicals should be expected to produce strand breaks in DNA. Such breaks can easily be detected if supercoiled plasmid DNA is used, since a single break will result in relaxed open circle plasmids and double strand breaks in linear DNA. These three forms of the plasmid can be separated by gel electrophoresis. When, for example, a plasmid of about 3 kb like Bluescript SK⁻ (BS-SK) is electrophoresed in a 0.7% agarose gel with TAE-buffer, the supercoiled form will migrate faster in the gel than the linear form, which migrates slightly faster than the open circle form. This assay can therefore be used to indicate and quantify strand break induction by test compounds and by modifying the exposure conditions to identify the mechanisms of strand break induction.

If not otherwise indicated 1 μg of BS-SK plasmid was treated with the test compound (100 μM) and various additives for 1 hr at 37° C in a total volume of 15 μl 10 mM Na-phosphate buffer (pH 7.0) and then gel electrophoreses. 4-chloro-2',3'- 3',4'- and 2',5'-dihydroxybiphenyl and the 2',5'-quinone were used as model compounds.

The first experiments were done without the addition of any metal ions. Under these conditions none of the tested PCB-metabolites alone induced strand breaks. A stepwise reduction of the pH did not influence this finding. Horseradish peroxidase (HRP, 0.001 U/μl) and H_2O_2 (0.2%) were then added the mixture for enzymatic oxidation of the dihydroxy-compounds. However, no induction of strand breaks was observed after 1 hr treatment.

Glutathione (GSH) could act as an activator for the quinone, since the resulting conjugate is a dihydroxy-metabolite that could oxidize to the corresponding quinone-conjugate with superoxide production. When the dihydroxy- and quinone-metabolites were tested in the presence of equimolar GSH, no induction of strand breaks was seen.

Only after 100 μM $CuCl_2$ was added to the dihydroxy-PCB/DNA mixture, a significant reduction of supercoiled plasmid and increase of open circle and linear plasmid was observed in the presence of test compound. In a comparative assay treatment with the para-quinone resulted in mainly open circle DNA, with the 2',5'-dihydroxy-metabolite in mostly linear plasmid; the activities of the 2 ortho-dihydroxy-metabolites were intermediate, with 3',4'- being slightly more damaging than 2',3'-. The effect of Cu(II) on PCB-DNA damage was concentration dependent with a threshold at about 80 μM $CuCl_2$ when 100 μM 2',5'-dihydroxy-metabolite was used. Cu(II) alone had no effect on the DNA. Other metal salts, Fe(III), Zn(II), Cd(II), and Mn(II) at 100 μM concentrations had no or only marginal effect on PCB-metabolite induced strand breaks. *In all following experiments 100 μM $CuCl_2$ was present in the treatment buffer, if not otherwise stated.*

The effect of the 2',5'-dihydroxy-metabolite was analyzed in more detail. The induction of strand breaks by this compound was concentration dependent. Even 25 μM 2',5'- induced strand breaks in 100% of the plasmid in the presence of 100 μM Cu(II). The severity of DNA damage was also time dependent: even after only 10 minutes of exposure some increase in open circle plasmid was observed. A doubling in time roughly doubled

the induced damage. The plan to enhance oxidation of dihydroxy-metabolites by HRP/H_2O_2 had to be abandoned, since the addition of H_2O_2 alone induced strand breaks in the presence of Cu(II).

The importance of Cu(II) for strand break induction was further shown by adding the divalent ion chelator EDTA. EDTA completely prevented strand break induction by 2',5'-dihydroxy at 100 μM concentrations. DETAPC, another chelator, was inactive at this concentration, however. A 10x excess of bathocuproine, a Cu(I) chelator nearly completely inhibited strand break induction.

GSH could influence the genotoxicity of our PCB-metabolites in 2 ways. By conjugation with quinones the resulting glutathione-dihydroxybiphenyl conjugate could undergo another round of oxidation with semiquinone, quinone and superoxide formation. GSH on the other hand is a redox partner that should reduce oxidative damage. GSH at 100 μM concentration reduced the DNA damage to about half with both compounds, the 2',5'-dihydroxy- and 2',5'-quinone-metabolite. No protection was seen with the 2 ortho-dihydroxy-metabolites.

To examine the involved species of oxygen radicals various scavengers and enzymes were added to the PCB-metabolite/Cu(II) incubation mixture. An equimolar concentration of the superoxide scavenger tiron was not protecting. Preliminary experiments with superoxide dismutase (1.2 U/μl) showed that the enzyme alone does not damage the DNA, but addition of 100 μM Cu(II) resulted in significant amounts of strand break. Further tests are planned. The enzyme catalase detoxifies H_2O_2 by converting it into water. Surprisingly catalase induced strand breaks in Cu(II)/phosphate buffer. When added to the 2,'5'-dihydroxy test compounds some protective effect of catalase was seen. With the ortho-dihydroxy compounds and para-quinone, it further increased the amount of strand breaks. Boiled and therefore enzymatically inactive catalase had no or only a very light protective effect with and without compounds.

Several compounds are hydroxyl radical scavengers. We tested DMSO and ethanol at various concentrations, mannitol, Na-benzoate, and thiourea at 100 μM - 200 mM. Adding 20% ethanol or DMSO to the incubation had only a marginal protective effect. Higher concentrations of DMSO did clearly protect the DNA from strand break induction. Na-benzoate at 100 mM or more resulted in disappearance (not due to increased strand breaks !) of DNA from the wells. Lower concentrations were not protective. Mannitol had no effect at any concentration tested. Only the hydroxyl radical scavenger thiourea inhibited strand break induction by the test compounds/Cu(II) and this effect was concentration dependent: equimolar amounts were ineffective, a 10x excess was slightly protective, with 10mM - 50mM the protection increased from strong to about complete.

Histidine and Na-azide are known scavengers for singlet oxygen. At 100 μM concentrations they did not protect the DNA from PCB/Cu(II) damage. Na-azide was tested at higher concentrations. With 2',5'-dihydroxy-metabolite as test compound and 25 mM Na-azide no linear plasmid was seen, with 50 - 200 mM an increase in supercoiled and decrease in open circle plasmid was observed, indicating a strong protective effect. As with GSH, Na-azide was not protective with the 2 ortho-dihydroxy-metabolites. 2',5'-dihydroxy-PCB induced strand breaks were also reduced with Tris, another singlet oxygen scavenger. Further experiments with the 2 ortho-metabolites and Tris are planned.

Table 2. **Strand break assay with 100 μM 4-Cl-2',5'-dihydroxybiphenyl**

Substance	Effect as/on	Concentration tested	Effect on DNA
Cu(II)	redox partner	100 μM	essential in this strand break assay
GSH	redox partner, cofactor	100 μM	protective
H_2O_2	cofactor	0.2%	induces strand breaks in Cu(II) medium
EDTA	chelator	100 μM	complete protection
DETAPAC	chelator	100 μM	no effect
phenanthroline	chelator	100 μM	destructive for DNA
bathocuproine	Cu(I) chelator	0.1 - 200 mM	complete protection with 1 mM
SOD	superoxide radical	1.2 U/μl	no effect
tiron	superoxide radical	100 μM	no effect at this conc.
catalase	H_2O_2	5 U/μl	protection of HQ induced damage
formic acid	hydroxyl radical	1%	destructive for DNA
DMSO	hydroxyl radical	7-53%	no effect <20%, protection at 20% or more
ethanol	hydroxyl radical	20%	no effect
mannitol	hydroxyl radical	0.1 - 200 mM	no effect
Na-benzoate	hydroxyl radical	0.1 - 200 mM	no protection, DNA vanishes (high conc.)
thiourea	hydroxyl radical	0.1 - 200 mM	some protection with 100 μM complete protection with 50 mM
histidine	singlet oxygen	100 μM	no effect at this conc.
Na-azide	singlet oxygen	0.1 - 200 mM	protective at >25 mM
Tris	singlet oxygen	5 - 50 mM	protective at all tested concentrations

Some of the assays above will be repeated with different concentrations and combinations. The results with the p-quinone and 2,3- and 3,4-dihydroxybiphenyl were similar to those above, but the activity was 2',5'- > 3',4'- > 2',3'- > p-quinone for 4-Chloro-biphenyl-metabolites. Preliminary data show less protection from strand break induction by ortho-dihydroxy metabolites with GSH and Na-azide. Catalase even seems to induce strand breaks with these compounds. Confirmation and further analysis of these results is in progress.

Our results with the NBT- and reduced cytochrome c assay show that superoxide is formed by autoxidation and enzymatic oxidation of dihydroxy metabolites of PCBs (see above). Superoxide, however, is not a potent inducer of DNA strand breaks. This was again confirmed in our first assays without metal ions. A transition metal partner is needed to convert superoxide to the strand break inducing ROS. Our results confirm that Cu(II) is most active compound in this regard.

Glutathione enhanced superoxide production by quinones in the NBT assay by reducing the compound to the dihydroxy-conjugate, which could undergo oxidation. In the strand break assay we did not see an enhancing effect. Rather a protective effect of GSH was observed with the 2',5'-dihydroxy-compound, but at equimolar concentrations not with the ortho-metabolites.

In the NBT assay we used enzymatic oxidation by peroxidases/H_2O_2 to enhance superoxide production by dihydroxy-metabolits. Since Cu(II) has to be included in the strand break assay and since Cu(II) converts H_2O_2 to the DNA damaging hydroxyl radical, we could not use enzymatic oxidation. This was not a problem, however, since the incubation time is longer and Cu(II) may act as a redox partner in the oxidation of the dihydroxy-compound to the semiquinone, as discussed by Flowers et al. (1997) for the oxidation of estrogen-catechols. This potential role of Cu(II) as redox partner is further suggested by the protective effect of the Cu(I) chelator bathocuproine.

Since SOD converts superoxide into H_2O_2 it is not surprising that we did not see a protective effect of this enzyme. The effects of catalase, an enzyme that detoxifies H_2O_2, where more puzzling and need further analysis.

Of the hydroxyl radical scavengers only thiourea and DMSO at high concentrations were protective. The other scavengers may be too weak or are DNA damaging themselves. The protective effect of Na-azide and Tris may indicate an involvement of singlet oxygen in the strand break induction by 4-Cl-2',5'-dihydroxybiphenyl, but further tests are needed. Interesting is again that Na-azide seems to have no protective effects when the ortho-metabolites are used for strand break induction.

7. DEVELOPMENT OF 8-OXODEOXYGUANOSINE (8-oxodG) AND ITS MEASUREMENT FOLLOWING TREATMENTS WITH PCBS

Free radical-mediated oxidative DNA damage has been implicated to play a role in chemical carcinogenesis. Because of its known mutagenicity, 8-OxodG has been frequently used as a benchmark oxidative DNA lesion. Our previous studies have demonstrated that nonenzymatic (copper-mediated) bioactivation of various mono-, di- and trichlorobiphenyl dihydroxy metabolites in the presence of calf thymus DNA results in significant elevation of 8-oxodG (400 – 4,000 8-oxodG/10^6 N) as compared to vehicle-treated DNA (100 8-oxodG/106 N) (Oakley *et al.*, 1996b). In this investigation a recently developed ^{32}P-postlabeling assay (Devanaboyina & Gupta, 1996) was used to measure 8-oxodG. The baseline 8-oxodG values determined by the ^{32}P-postlabeling assay for calf thymus and various rat tissues is generally 10-20-fold higher than the values reported by others by a commonly used but less sensitive HPLC-ECD procedure (Devanaboyina & Gupta, 1996). Despite these differences noted in the two procedures, the results obtained in the nonenzymatic activation of PCB metabolites are unlikely to be influenced because of relatively large differences between dihydroxy PCB treatment and vehicle treatment. However, this methodology appeared unsuitable to determine smaller differences as expected for *in vivo* situation until it was established that 8-oxodG values were not increased during the workup. The ^{32}P-postlabeling methodology was therefore scrutinized systematically as described below:

The ^{32}P-postlabeling method for measuring 8-oxodG comprises enzymatic degradation of DNA (2-5 μg) to 3'-monophosphates of normal and oxidized nucleosides, 5'-^{32}P-labeling of the digest (100 ng) in the presence of molar excess of [γ-^{32}P]ATP (>300 Ci/mmol; ~20 μM) and T4 polynucleotide kinase at room temperature for 45-60 min, conversion of the 3',5'-bisphosphates to 5'-monophosphates by

incubation with nuclease P1 (0.1 μg/ul) and 0.1 mM $ZnCl_2$ at room temperature for 45-60 min, and finally separation of [5'-^{32}P]8-oxodG by 2-directional PEI-cellulose TLC (Devanaboyina & Gupta, 1996). There are two enzymatic steps, i.e., the polynucleotide kinase phosphorylation and nuclease P1 dephosphorylation, during which the unadducted nucleotides, primarily dGp, constantly undergoes exposure to the isotope ^{32}P and any potential metal ion contaminants which are potentially capable of producing 8-oxodG from the parent nucleotide. Two studies were therefore performed with rat lung DNA as an example to determine if 8-oxodG may be produced artifactually during these enzymatic reactions:

Study 1. Effect of the isotope ^{32}P during T4 polynucleotide kinase phosphorylation. Following enzymatic digestion of rat lung DNA, the digest was ^{32}P-labeled under standard conditions, except that the incubation was performed for different periods, i.e., 1, 3, 7 and 23 h. The labeled digests were then treated with nuclease P1 under standard conditions (60 min. incubation), followed immediately by chromatography to arrest the reaction. Measurement of the radioactivity in 8-oxodG spot revealed a time dependent increase in 8-oxodG levels (55, 39, 71 and 58/10^6 N after 1, 3, 7 and 23 h, respectively) (Table 3).

Table 3: Effect of the isotope ^{32}P during T4 polynucelotide kinase phosphorylation on 8-oxodG production.

Incubation time (h)	8-oxodG/10^6 N
1	55.4 ± 5.2
3	38.7 ± 4.8
7	71.0 ± 9.0
23	58.2 ± 12.7

8-OxodG values represent mean ± S.D. (n=3).

These results suggest that exposure of unadducted dG to ^{32}P results in small but insignificant production of 8-oxodG and that under the standard working protocol, it is unlikely to have a significant contribution of artifactual 8-oxodG.

Study 2. Effect of the isotope ^{32}P during nuclease P1 dephosphorylation reaction. Following ^{32}P-labeling of the DNA digest under standard conditions, the labeled digest was treated with nuclease P1 for different periods, i.e., 1, 3, 7 and 23 h, followed immediately by chromatography to arrest the reaction. Measurement of the 8-oxodG radioactivity revealed a time-dependent increase in 8-oxodG levels (50, 57, 110 and 134/10^6 N) after 1, 3, 7 and 23 h, respectively) suggesting that exposure of

unadducted dG to the isotope and nuclease P1 results in 8-oxodG production (Table 4).

Table 4: Effect of the isotope ^{32}P during nuclease P1 dephosphoprylation reaction on 8-oxodG production.

Incubation time (h)	8-oxodG/10^6 N
1	50.2 ± 6.4
3	57.0 ± 8.6
7	109.5 ± 5.2*
23	134 ± 19.0*

8-OxodG values represent mean ± S.D. (n=3).
***P<0.001 (Student t test) compared to 1 h time point.**

This increase could be due to Zn++, a cofactor for the nuclease P1, other contaminating transition metals present in the sodium acetate buffer or some peroxidase-like activity as contaminant present in the commercial preparation of nuclease P1. The effect of various transition metals on the 8-oxodG production during the workup is currently in progress.

It is apparent from Studies 1 and 2 that 8-oxodG is produced from the parent nucleotide by exposure to [γ-^{32}P]ATP and/or nuclease P1-cofactor mixture but it is difficult to assess the actual amount of 8-oxodG that may be produced under the assay conditions. It therefore became necessary to develop strategies that will allow enrichment of 8-oxodGp by removal of normal nucleotides prior to ^{32}P-labeling, as described below.

Study 3. Enrichment of 8-oxodGp. Modified DNA containing high levels of 8-oxodG (1,000 – 10,000 8-oxodG/10^6 N) were prepared to develop the enrichment strategy. The reference DNA were prepared by treatment of calf thymus DNA with $^{\bullet}$OH radicals produced in a typical Fenton reaction, i.e., in the presence increasing concentration of $FeSO_4/H_2O_2$. The purified DNA preparations were analyzed to determine the 8-oxodG levels by the published ^{32}P-postlabeling method. A previously reported enzymatic adduct enrichment procedure in which normal nucleoside 3'-monophosphates are selectively converted to their nucleosides, while adducted nucleotides remain unhydrolyzed, was however found to be unsuitable because 8-oxodGp was sensitive to 3'-dephosphorylation by nuclease P1 treatment. We then took advantage of published PEI-cellulose TLC system (Devanaboyina & Gupta, 1996) in which 5'-labeled normal nucleotides readily separate from 5'-^{32}P-labeled 8-oxodG

when the thin layer chromatogram was developed in solvents of relatively low pH (1-2 M formic acid).

We focused on two major parameters to develop this TLC enrichment scheme: (1) Separation of normal nucleotides from 8-oxodGp and (2) Elution of 8-oxodGp from the thin layer. The first parameter was standardized by employing 5'-^{32}P-labeled normal deoxynucleosides and 8-oxodG as tracers and selecting varying concentrations of formic acid (0.02 – 0.8 M) and varying lengths of PEI-cellulose chromatogram. The TLC development under which normal nucleotides migrated sufficiently away from 8-oxodGp (making the latter free from normal nucleotides by >99.9%) and that 8-oxodGp stayed at or close to the origin were selected. This objective was accomplished when the tracer nucleotides were chromatographed onto a 10 cm-long PEI-cellulose PEI-cellulose sheet and the development was in 0.2-0.4 M formic acid onto a Whatman #17 paper wick. The second parameter was standardized by first chromatographing 5'-^{32}P-labeled 8-oxodG on a PEI-cellulose thin layer and then testing a variety of eluents (4 M pyridinium formate, 0.2 - 2 M triethylammonium acetate, 1 M ammonium hydroxide, etc.) to elute the labeled 8-oxodG. 1-2 M triethylammonium acetate was found to be a highly desirable eluent because it resulted in almost complete recovery and moreover the salt could be removed by repeated lyophilization.

The standardized TLC enrichment conditions were then applied to determine basal levels of 8-oxodG in calf thymus DNA. Two to 5 μg DNA digest was enriched by PEI-cellulose TLC and the origin area containing 8-oxodGp was eluted and lyophilized. After dissolving in appropriate volume of water, an aliquot (500 – 1000 ng DNA) was ^{32}P-labeled in the presence of carrier-free [γ-^{32}P]ATP (>1,500 Ci/mmol; 50 μCi; <2 μM), converted to 5'-monophosphates by treatment with nuclease P1 and the ^{32}P-labeled 8-oxodG was separated by published 2-directional PEI-cellulose TLC. This new procedure resulted in up to a 100-fold higher 8-oxodG radioactivity than found in the non-enrichment procedure because the modified method allowed the use of a 5-10-fold higher amount of DNA and a 10-fold higher specific activity of ATP. Thus, not only the assay sensitivity was enhanced by 2-orders of magnitude, but it also increased signal to noise to 50:1 from 3-5:1 generally observed in the non-enrichment assay. Calf thymus DNA standards containing varying degree of 8-oxodG were also successfully analyzed by this enrichment procedure. Although we have not as yet fully established the 8-oxodG recovery, preliminary assessments indicate a 65%-85% recovery.

Induction of 8-oxodG in tissue DNA of rats treated with low chlorinated PCBs.

A preliminary *in vivo* experiment was conducted to determine if oxidative DNA damage is induced following treatment with selected mono-, di- and tri-, tetra- and hexa-chlorobiphenyls. Conditions for the animal treatments were described in Section 3 above. DNA was isolated from frozen liver by a solvent extraction procedure involving isolation of crude nuclei, removal of RNA and protein by digestions with RNases and proteinase K and solvent extractions, followed by precipitation of DNA with ethanol.

DNA (10 μg) was then enzymatically hydrolyzed and 8-oxodGp were enriched under the standardized conditions. An aliquot of the enriched 8-oxodGp (1,000 ng DNA) was ^{32}P-labeled and separated by 2-directional PEI-cellulose TLC following conversion to 5-monophosphates by treatment with nuclease P1. 8-oxodG spot was detected by InstantImager (Figure 4).

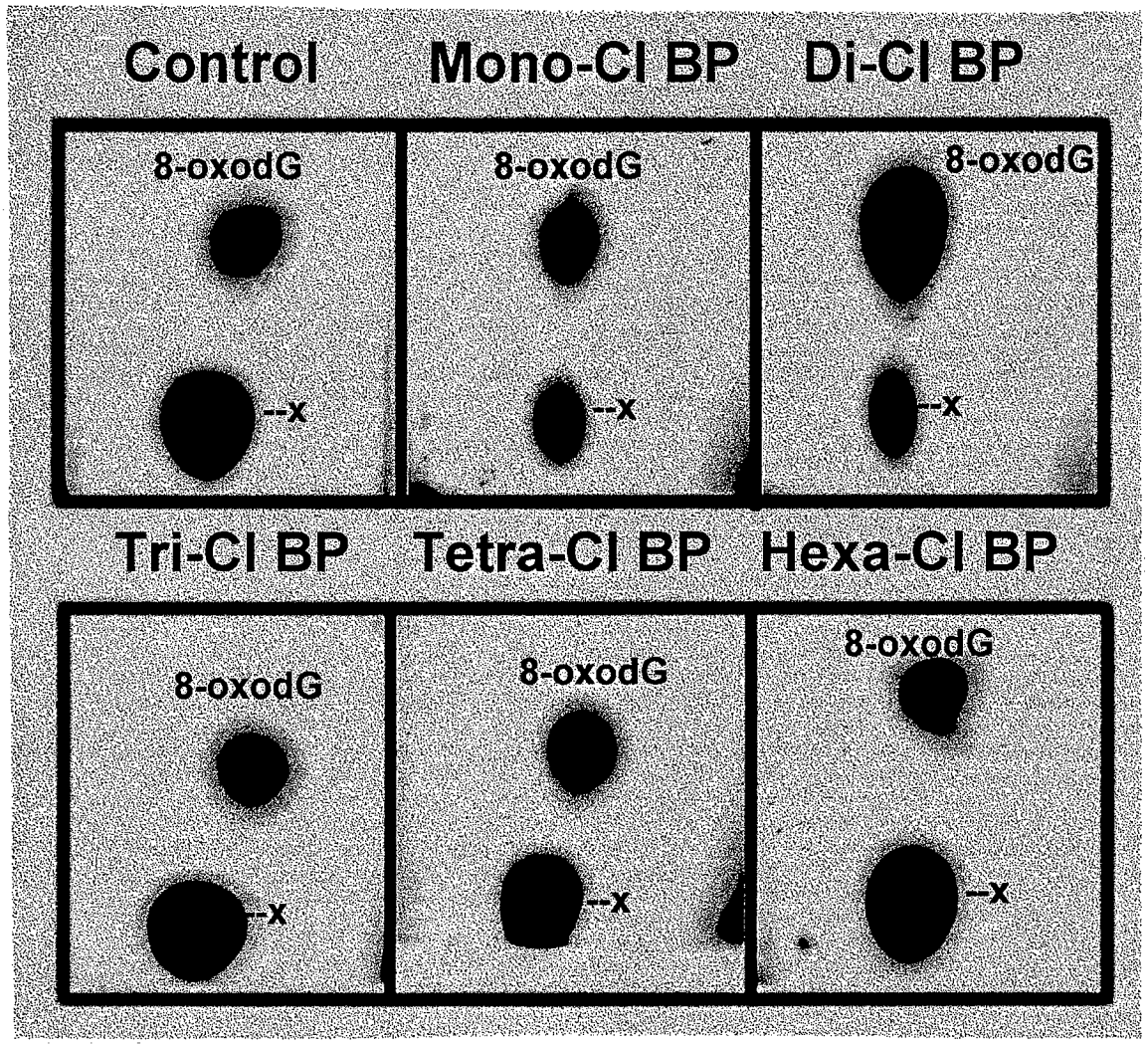


Figure 4: ^{32}P-Postlabeling maps for 8-oxodG production in the liver of rats treated with different PCBs. Autoradiogams represent 500 ng DNA and 3 h exposure at room temperature using Cronex 4 X-ray film. 'X' denotes contaminant spot.

An aliquot of diluted DNA digest (2 ng) was also ^{32}P-labeled in parallel and the labeled nucleotides were separated by 1-directional PEI-cellulose TLC and detected by InstantImager. Relative adduct labeling (RAL) of 8-oxodG was then calculated by measurement of the adduct radioactivity in 8-oxodG and normal nucleotides as described elsewhere. As compared to vehicle-treated liver DNA (13.3 ± 0.96 8-oxodG/10^6 N) only 3,4-dichlorobiphenyl (24 ± 4.8 8-oxodG/10^6 N) resulted in up to 2-fold increase in 8-oxodG levels (Table 5).

Table 5: 8-OxodG production in the rat liver after PCB exposure

Treatments	8-OxodG/10^6 N
Corn oil	13.3 ± 0.96 (n=4)*
4-Cl BP	7.8 ± 0.8 (n=2)
3,4-di Cl BP	24.0± 4.8 (n=2)
2,4,4'-tri Cl BP	11.8 ± 4.3 (n=4)
3,3',4,4'-tetra Cl BP	11.7 ± 3.2 (n=3)
2,2',4,4',5,5'-hexa Cl BP	9.3 ±0.93 (n=3)

8-OxodG values represent mean ± S.D. Asterisk shows analytical replicates from 2 animals and the others are biological replicates.

These results suggest that 8-oxodG can be readily detected and quantitated in rat tissues by an improved ^{32}P-postlabeling assay. Because of the small tissue specimens used in this study, it remains to be determined if the increase in 8-oxodG levels found after treatment with 3,4-dichlorobiphenyl is significant or not. Experiments with a larger number of rats both female and male, and various tissues, including breast epithelium, have been planned to further investigate the *in vivo* induction of oxidative DNA damage.

CONCLUSIONS

1. Microsomes prepared from human breast tissue are capable of catalyzing the hydroxylation of lower chlorinated PCBs in the presence of NADPH. Three mono- and three di-hydroxy metabolites of 4-chlorobiphenyl have been tentatively identified.

2. Several mono- and di-hydroxy metabolites of PCBs have been tested in a human breast cell assay, the MCF-7 Focus assay (in collaboration with Dr. John Gierthy), in order to test their estrogenicity (antiestrogenicity). Our results indicate that the estrogenicity of hydroxylated PCB metabolites is mediated through an estrogen receptor and modulated by the position of the hydroxyl group. The most active (estrogenic) PCB metabolites are para hydroxylated.

3. To detect alterations in nuclear proteins that may potentially explain the tumor promoter activity of PCBs, liver nuclear extracts were analyzed by electrophoretic mobility shift assays (EMSAs). 4-Chloro-, 2,4,4'-trichloro, and 2,2',4,4',5,5'-hexachlorobiphenyl treatment of Sprague-Dawley rats resulted in a large increase in protein binding to a consensus AP-1 element. Further analysis of the proteins binding to the AP-1 consensus sequence using antibodies specific for c-FOS, JUN-D and JUN-B indicate that the protein composition consists of JUN-B proteins. EMSA analysis of STAT binding activity to a consensus GAS element was compared in the various PCB

treated liver nuclear extracts. STAT binding activity was 8 to 10-fold higher in nuclear extracts from 2,4,4'-trichloro- and 2,2',4,4',5,5'-hexachlorobiphenyl-treated animals.

4. Ample evidence is presented that during the oxidation of di-hydroxy PCB metabolites, in several enzyme systems, superoxide may be produced. Thius was detected by monoriting the reduction of Nitro Blue Tetrazolium (NBT) spectrometrically.

5. To determine if PCB quinones are able to participate in redox events, we incubated liver microsomes, PCB quinones, a NADPH regenerating system, and cytochrome c as an *in vitro* test system. We then measured the rate of cytochrome c reduction as an indicator of superoxide production. To confirm that superoxide free radicals were in fact being produced, we performed all experiments in the presence and absence of superoxide dismutase (SOD). The rate of cytochrome c reduction was decreased in the presence of SOD for all PCB quinones except one, indicating that superoxide was a product in the PCB quinone-catalyzed redox cycling.

6. Strand Breaks are induced by PCBs metabolites when transition metals are present. Hydroxyl radical scavengers and singlet oxygen scavengers protect.

7. Considerable effort has been expended to establish an assay for the determination of oxidized DNA bases, especially 8-oxodeoxyguanosine (8-oxodG). Results presented show that 8-oxodG can now be readily detected and quantified in rat tissues by an improved ^{32}P-postlabeling assay. Experiments with both female and male rats, and various tissues, including breast epithelium, are planned to further investigate the *in vivo* induction of oxidative DNA damage.

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